Journal of Medical Virology 58:121–126 (1999)

Quantification of Hepatitis C Virus by TaqMan PCR: Comparison With HCV Amplicor Monitor Assay

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The quantitation of serum levels of hepatitis C virus RNA in chronic hepatitis C has been regarded as one of the most important indicators for the outcome of interferon therapy. A new method was used for quantitating the copy number of hepatitis C virus RNA using TaqMan polymerase chain reaction and for comparing the ability and usefulness of this assay with Amplicor Monitor assay in 138 patients. The detection range of hepatitis C virus RNA by TaqMan polymerase chain reaction was from 2×10^3 to 2×10^8 copies/ml. Hepatitis C virus RNA was detectable in 128 cases (92.8%) and undetectable in 10 cases (7.2%) by this method. The RNA levels measured by Amplicor Monitor assay correlated significantly with those measured by TaqMan polymerase chain reaction assay and the sensitivity of the two assays was almost equal. Thus, TaqMan polymerase chain reaction assay appears sufficiently sensitive for the evaluation of hepatitis C virus RNA and would be useful for the diagnosis and management of hepatitis C virus infection. ***J. Med. Virol. 58:121–126, 1999.***

KEY WORDS: hepatitis C virus RNA; polymerase chain reaction; viral load

INTRODUCTION

Patients with hepatitis C virus (HCV) infection usually develop chronic hepatitis, and some eventually develop cirrhosis of the liver and hepatocellular carcinoma [Alter et al., 1989; Kiyosawa et al., 1990]. Interferon (IFN) has been shown to normalize serum alanine aminotransferase (ALT) levels in some patients with chronic hepatitis C [Davis et al., 1989; Di Bisceglie et al., 1989]. Although approximately 50%–60% of treated patients respond, half of the patients relapse after cessation of the therapy [Davis et al., 1989; Di Bisceglie et al., 1989]. The quantitation of serum levels of HCV RNA in chronic hepatitis C has been regarded as one of the most important indicators for the outcome of IFN therapy, since a sustained response can be expected in patients with a low virus load [Lau et al., 1993]. Several assays have been developed for evaluation of the HCV RNA load using polymerase chain reaction (PCR) or a hybridization procedure such as competitive reverse transcription-PCR (CRT-PCR) [Kato et al., 1990, 1993], combined reverse transcription-PCR assay (Amplicor-HCV Monitor; Roche Molecular Systems, Branchburg, NJ) [Young et al., 1993, 1995; Roth et al., 1996], and branched chain DNA probe assay (bDNA probe assay; Chiron, Emeryville, CA) [Lau et al., 1993; Detmer et al., 1996].

Recently, significant progress has been made in the quantitation of target nucleic acid molecules with the introduction of the "TaqMan" fluorescence energy transfer assay [Holland et al., 1991; Gibson et al., 1996; Heid et al., 1996; Morris et al., 1996]. This assay uses a nucleic acid probe complementary to an internal segment of the target DNA. The probe is labeled with two fluorescent moieties with the property that the emission spectrum of one overlaps the excitation spectrum of the other. As a result, the emission of the first fluorophore is largely quenched by the second. The probe is introduced during PCR. When the PCR product is made, the probe becomes susceptible to degradation via the 5′–3′ endonuclease activity of *Taq* DNA polymerase that is specific for DNA hybridized to the template (TaqMan activity). Nucleolytic degradation of the probe allows the two fluorophores to separate in solution, which reduces quenching and increases the intensity of emitted light. Successive PCR cycles result in exponential amplification of the PCR product and fluorescence intensity. PCR products are detected within minutes of completion of the PCR reaction by monitoring the increase in fluorescence of the dye-labeled probe. TaqMan PCR was used for quantitative measurement of HCV RNA, and the usefulness of this assay was compared with the Amplicor Monitor assay.

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Accepted 26 October 1998

MATERIALS AND METHODS

Patients

One hundred thirty-eight patients with chronic hepatitis C (75 men and 63 women; mean age, 50.9 ± 14.5 years; range, 13–82 years) who visited Chiba University Hospital between June 1996 and May 1997 were examined in this study. The diagnosis of chronic hepatitis C was made on the basis of elevated serum ALT levels (102.0 ± 75.2 IU/L) for more than 6 months and positivity for second-generation anti-HCV antibody testing by enzyme immunoassay (Dainabot, Tokyo, Japan). Fifty-six of the patients were also biopsy-proven for chronic hepatitis. Twenty-one patients had a history of unsuccessful IFN treatment. Eight patients were treated with IFN after these assays.

Extraction of RNA From the Serum

RNA was extracted from the serum according to the guanidine thiocyanate method as previously described [Kato et al., 1990]. To extract RNA, 50 μl of serum were added to 400 μl of 4.2-M guanidine thiocyanate, 0.5% sodium-N-laurylsarcosine, and 25-mM Tris-HCl (pH 8.0) and mixed well. Then the mixture was extracted twice with phenol/chloroform and once with chloroform. Forty-μl of 3-M sodium acetate and 10 μg of dextran were added to the aqueous phase, and the RNA was precipitated with 900 μl of absolute ethanol. After storage at −70°C for 30 min, the RNA was pelleted, dried in air, and dissolved in 10 μl of distilled water.

Preparation of Control HCV RNA

HCV RNA was extracted from an anti-HCV–positive patient's serum as described above. To construct RNA control, HCV cDNA fragment was amplified with a set of primers (5′-GGGCGAATTCCCACCATAGATCACTCCCTGT-3′ and 5′-TTTAGGATTTGAGCTCATGATGCACGGTCT-3′) using RT-PCR. The amplified product was digested with *Eco*RI and *Sac*I (Takara Shuzo, Kyoto, Japan) and cloned into pGEM- 3Zf(+) plasmid (Promega, Madison, WI) by standard technique. In vitro, RNA transcription from *Hinc*II-digested pGEM3Zf(+) was performed by use of T7 RNA polymerase according to the manufacturer's instructions (Riboprobe; Promega). The optical density of the transcribed control RNA was measured and the amount of control RNA was determined. To assess the quality of the preparation, a control HCV RNA transcript, 358 base fragment in length, was electrophoresed on 1.5% formaldehyde gel and stained by SYBR Green I Nucleic Acid Gel Stain (Takara Shuzo). The preparation of the HCV RNA transcript used in this study consisted of over 95% full-length transcripts.

Measurement of HCV RNA by TaqMan PCR Assay

To measure HCV RNA, TaqMan EZ RT-PCR kit (PE Applied Biosystems, Foster City, CA) was used. Forward and reverse primers (5′-TAGTGGTCTGCGGAACCGGT-3′ and 5′-TGCACGGTCTACGAGACCTCC-3′) that amplify a 199-bp segment of the 5′ noncoding region of HCV RNA and a dual fluorophore-labeled probe [5′-(6-carboxy-fluorescein)-TGCCTGATAGGGTGCTTGCGAGTGCC-(6-carboxy-tetramethyl-rhodamine)-3′] were designed.

Fifty μl of PCR mixture contained TaqMan EZ buffer (PE Applied Biosystems), 4-mM $Mn(OAc)_2$, 0.2 mM each of dATP, dCTP, and dGTP, 0.4-mM dUTP, 0.2-μM forward and reverse primers, 0.1-μM dual fluorophore-labeled probe, 7.5-U *Thermus thermophilus* (r*Tth*) DNA polymerase, 0.5-U AmpErase uracil N-glycosylase (UNG), and template RNA. RT-PCR amplification was started with 2-min incubation at 50°C for digestion of possible contamination with AmpErase UNG, then 30min at 65°C for reverse transcription with r*Tth* DNA polymerase, then 5 min at 95°C for deactivation of UNG followed by 50 cycles of denaturation at 95°C for 20 sec and annealing/extension at 65°C for 1 min. All reactions were performed in an Applied Biosystems PRISM 7700 Sequence Detector (PE Applied Biosystems), which contained the Gene Amp PCR System 9600. Reaction conditions were programmed on a Power Macintosh 7100 (Apple Computer, Santa Clara, CA) linked directly to the sequence detector. Analysis of data was also performed on the Macintosh computer. Data were collected and analyzed using the software developed by PE Applied Biosystems.

Measurement of HCV RNA by Amplicor Monitor Assay

HCV RNA in the serum was quantified with the Amplicor-HCV Monitor assay kit (Nippon Roche, Tokyo, Japan). The Amplicor Monitor assay was performed with 100 μl of serum according to the procedure used for quantitation of the human immunodeficiency virus [Mulder et al., 1994]. The principle of this assay has been described previously [Young et al., 1993, 1995; Roth et al., 1996]. It involves three steps: sample preparation after adding a constant amount of internal quantitative standard RNA; RT-PCR with r*Tth* DNA polymerase; and detection of amplified products on Amplicor microwell plates coated with albumin-conjugated immobilized probes and an internal quantitative standard amplicon-specific DNA probe.

Serotyping of HCV

HCV serotypes were determined by a serotyping assay according to the method of Tsukiyama-Kohara et al. [1991] and Tanaka et al. [1994]. This assay recognizes HCV serotypes 1 and 2, which are the most prevalent types in Japan. In this analysis, HCV serotype 1 corresponds to genotypes 1a and 1b, and HCV serotype 2 corresponds to genotypes 2a and 2b of the classification of Simmonds et al. [1993].

Statistics

Data are presented as mean values ± standard deviation. Statistical analysis was performed by Spearman's rank analysis. The level of statistical significance was set at $P < 0.05$.

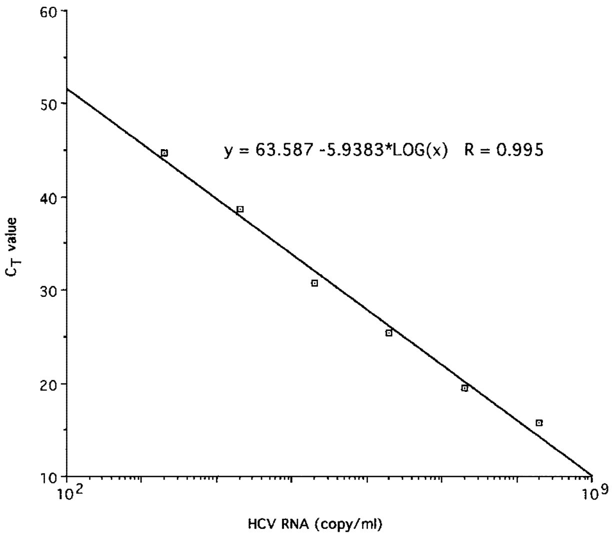

Fig. 1. Colinearity of dilution and assay range of HCV RNA. Tenfold serial dilutions of control RNA were prepared in duplicate, reverse transcribed, and amplified using sequence detection. Mean C_T values are plotted against the relative copy numbers.

RESULTS

HCV Serotype

Of the 138 cases, 91 (65.9%) were HCV serotype 1, 41 (29.7%) were HCV serotype 2, and 6 (4.3%) were a mixture of HCV serotypes 1 and 2.

Sensitivity of HCV RNA Detection by TaqMan PCR

Serial 10-fold dilutions of control HCV RNA were made, ranging from 2×10^8 copies/ml to 20 copies/ml (10^7 copies to 1 copy/tube). Threshold cycles (C_T) were calculated from observed fluorescence as described [Gibson et al., 1996; Heid et al., 1996] and plotted against the relative amount of control RNA (Fig. 1). A significant first-degree correlation was found between the C_T value and log of initial target copy number ($r = 0.995$, Fig. 1). The limiting number for HCV RNA detection by our assay system was 2×10^3 copies/ml and the detection range was up to 2×10^8 copies/ml. When 1×10^4–1×10^9 copies/ml of these control HCV RNAs were measured by Amplicor Monitor assay as the reference sample, the values were determined as 1×10^3–6.6×10^6 copies/ml. There was a linear correlation between the values obtained by Amplicor Monitor assay and by TaqMan PCR assay ($r = 0.993$). Thus, the values of HCV RNA by Amplicor Monitor assay were 1/10–1/100 lower than the results obtained by TaqMan PCR assay, and higher levels of HCV RNA were relatively more underestimated by Amplicor Monitor assay compared to the TaqMan PCR assay.

Quantitation of HCV RNA by TaqMan PCR Method

In TaqMan PCR, quantitation of the amount of target in the 138 unknown samples was accomplished by measuring C_T values. The copy numbers were determined based on the standard curve drawn by the known amount of control HCV RNA as described above. The determination of HCV RNA by TaqMan PCR was performed in duplicate. The correlation coefficient of these duplicate determinations was $r = 0.949$. In this study, the amount of HCV RNA in the serum samples was defined as the mean of the duplicate data.

TABLE I. Comparison of Serum HCV RNA Detection by the Two Different Quantitative Methods[a]

Amplicor/TaqMan	+/+	–/+	+/–	–/–
Serotype 1	86	0	2	3
Serotype 2	35	3	1	2
Serotype 1 + 2	4	0	0	2
Total	125	3	3	7

[a]+, detectable by the method; –, undetectable by the method.

Of the 138 cases examined, HCV RNA was detectable in 128 cases (92.8%) and undetectable in 10 (7.2%) (Table I). It was detectable in 86/91 (94.5%) of serotype 1, in 38/41 (92.7%) of serotype 2, and in 4/6 (66%) of serotype 1 + 2 (Table I). The range of HCV RNA determined by TaqMan PCR was up to 1.1×10^8 copies/ml in serotype 1, up to 6.5×10^7 copies/ml in serotype 2, and up to 1.2×10^8 copies/ml in serotype 1 + 2.

Quantitation of HCV RNA by Amplicor Monitor Assay

Of the 138 cases examined, HCV RNA was detectable in 128 (92.8%) and undetectable in 10 (7.2%) (Table I). It was detectable in 88/91 (96.7%) of serotype 1, in 36/41 (87.8%) of serotype 2, and in 4/6 (66%) of serotype 1 + 2 (Table I). The range of HCV RNA determined by Amplicor Monitor assay was up to 1.8×10^6 copies/ml in serotype 1, up to 1.2×10^6 copies/ml in serotype 2, and up to 4.1×10^5 copies/ml in serotype 1 + 2.

Comparison of the Detection of HCV RNA by TaqMan PCR Assay and Amplicor Monitor Assay

In 10 (7.2%) of the total 138 cases, HCV RNA level was lower than the detection limit by TaqMan PCR assay, as described above. Seven undetectable cases were also undetectable by Amplicor Monitor assay, but two of the cases were positive by Amplicor qualitative assay (Nippon Roche). The five other cases were positive for second-generation anti-HCV antibody testing by enzyme immunoassay kit and ALT levels were elevated for more than 6 months. The loads of HCV RNA in these five cases were under the detection limits by these HCV RNA methods. The values of the three cases in whom HCV RNA was detectable by TaqMan PCR assay but not by Amplicor Monitor assay were 8.1×10^3, 3.6×10^4, and 4.8×10^5 copies/ml (all serotype 2) (Table I). In addition, there were also three cases in whom HCV RNA was detectable by Amplicor Monitor assay but not by TaqMan PCR assay, and the values were 1.2×10^3, 3.6×10^3, and 4.7×10^3 copies/ml (two cases were serotype 1 and one case serotype 2) (Table I).

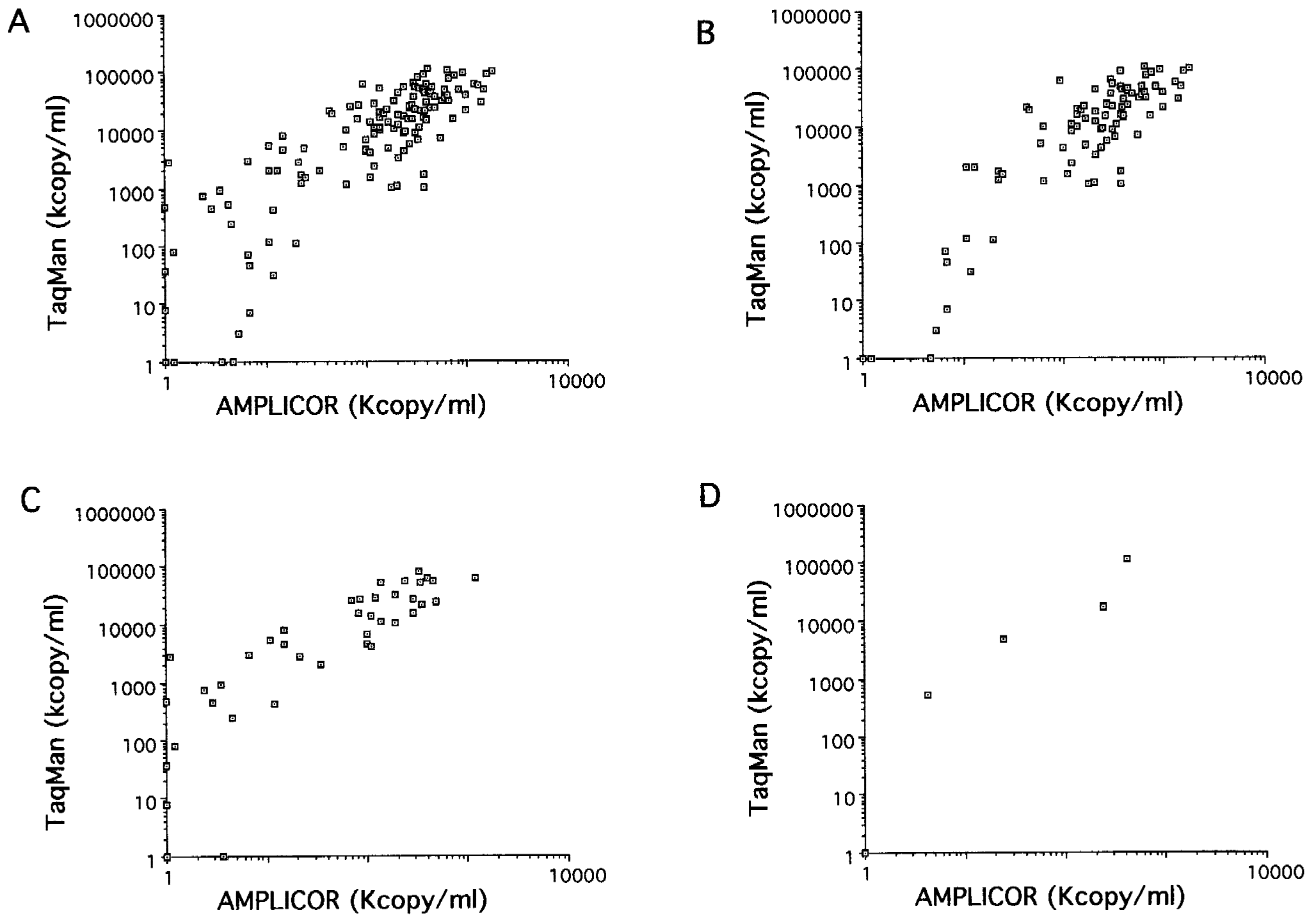

Fig. 2. Relationship between HCV RNA levels measured by Amplicor Monitor and TaqMan PCR assays in 138 samples (**A**). HCV serotypes were determined by a serological typing assay: (**B**) serotype 1; (**C**) serotype 2; (**D**) serotype 1 + 2. The concentration of HCV RNA measured by TaqMan assay is significantly correlated with that measured by Amplicor Monitor assay (Spearman's rank analysis; A, r = 0.808, $P < 0.0001$; B, r = 0.782, $P < 0.0001$; C, r = 0.892, $P < 0.0001$; D, r = 1.000, $P < 0.05$).

As shown in Figure 2, HCV RNA levels measured by the Amplicor Monitor assay correlated significantly with those measured by the TaqMan PCR assay (Fig. 2A, r = 0.808, $P < 0.0001$). Positive correlations between HCV RNA levels measured by these assays were obtained for HCV serotype 1 (Fig. 2B, r = 0.782, $P < 0.0001$), serotype 2 (Fig. 2C, r = 0.892, $P < 0.0001$), and serotype 1 + 2 (Fig. 2D, r = 1.000, $P < 0.05$). The actual copy numbers determined by Amplicor Monitor assay were considerably lower than those determined by TaqMan PCR assay. Especially in cases with serotype 2, the copy numbers determined by Amplicor Monitor assay were 10–100 times lower (Fig. 2).

DISCUSSION

Measurement of serum concentration of HCV RNA is essential for the treatment of chronic hepatitis C because the concentration is an effective predictive marker for the response of the patient to IFN therapy [Lau et al., 1993]. In this study, we demonstrated that HCV RNA was quantitatively detectable by the use of TaqMan PCR. The clinical usefulness of the TaqMan PCR method was demonstrated.

Hitherto various assays based on different principles were developed for the quantitative measurement of HCV RNA [Kato et al., 1993; Lau et al., 1993; Detmer et al., 1996; Roth et al., 1996]. These assays have various levels of sensitivity. One of these, the CRT-PCR assay, is sensitive and can quantify HCV RNA with a wide-ranging spectrum. However, this type of assay requires nested amplification to achieve the desired level of sensitivity and thus may have the risk of carry-over contamination with molecules amplified in a previous PCR. The bDNA probe assay, which is not based on the PCR technique, was developed as a simple assay kit for clinical use. This assay has a lower risk of contamination than assays using the PCR technique, but is less sensitive [Lau et al., 1995; Shiratori et al., 1997a]. The Amplicor Monitor assay is a combined RT-PCR assay in which RT and PCR steps are performed under a single set of conditions [Roth et al., 1996]. The risk of contamination in this assay is greatly reduced by UNG and dUTP steps [Longo et al., 1990; Udaykumar et al., 1993] incorporated prior to thermal cycling to ensure selective destruction of potential carry-over from previous amplification reactions.

TaqMan PCR is also performed using a single-tube, single-enzyme system for reverse transcription of RNA

to cDNA and amplification using the PCR process. Reverse transcription and DNA polymerization processes take place without the addition of subsequent enzymes or buffers. Direct detection of the RT-PCR product with no downstream processing is accomplished by monitoring the increase in fluorescence of a dye-labeled DNA probe [Gibson et al., 1996]. Because this assay involves fluorescence measurements that can be performed without opening the PCR tube, the risk of carry-over contamination is greatly reduced. This assay does not need intensive labor and is easily automated. Thus, this method makes it possible to analyze hundreds of samples per day if necessary.

To improve the precision of the data, the C_T value is used for quantification of the samples by the TaqMan method. The early cycles of PCR are characterized by an exponential increase in target amplification. As reaction components in reaction tubes are limiting, the rate of target amplification decreases until a plateau is reached, and there is little or no net increase in PCR product after multiple amplification cycles. The sensitive fluorescence detection allows the C_T values to be observed when PCR amplification is still in the exponential phase. During the exponential phase, none of the reaction components is limiting; as a result, C_T values are very reproducible for reactions with the same starting copy number. This is the main reason why C_T is more reliable for measurement of the starting copy number than endpoint measurement of the amount of accumulated PCR product. This leads to greatly improved precision in the quantitation of minimal DNA and RNA.

Regarding the sensitivity and the ranges of the measurement, serum HCV RNA levels were determined by several assay methods, but the absolute number of HCV RNA copies per milliliter varies from assay to assay [Ichijo et al., 1997; Shiratori et al., 1997a, 1997b]. The sensitivity of the Amplicor Monitor assay was about 10% less than that of the CRT-PCR assay, but was better than the bDNA probe assay by about 20% [Ichijo et al., 1997]. Similarly, the effective quantitative range of the Amplicor Monitor assay was about 10 times narrower than that of the CRT-PCR assay, but about 10^2 times wider than that of the bDNA probe assay [Ichijo et al., 1997]. The concentration of HCV RNA measured by the Amplicor Monitor assay was significantly correlated with those measured by both the CRT-PCR and bDNA probe assays [Ichijo et al., 1997; Shiratori et al., 1997a, 1997b].

In this study, TaqMan PCR had a very large dynamic range of starting target molecules. The concentration of HCV RNA measured by TaqMan PCR assay was significantly correlated with that measured by Amplicor Monitor assay. Although there is no gold standard for the evaluation of the amount of HCV RNA, HCV RNA levels measured by TaqMan PCR assay were around 10–100-fold higher than those measured by Amplicor Monitor assay (Fig. 2A). The principles for the determination of HCV RNA level were considerably different between these assays (Amplicor principle, Roth et al. [1996]; TaqMan principle, Gibson et al. [1996]), and therefore the difference in values might be a result of these differences. In addition, from the results of the Amplicor Monitor assay on reference control HCV RNA for TaqMan PCR assay, another reason for the difference might be the difference of the control RNAs of the two methods. Unfortunately, the control RNA for Amplicor Monitor assay could not be determined by the TaqMan PCR assay because the sequence of the internal quantitative standard was different from the sequence of HCV RNA between the primers used in the Amplicor Monitor assay. It should also be noted that the HCV RNA levels measured by the Amplicor Monitor assay were around 10–20-fold lower than those measured by CRT-PCR according to Shiratori et al. [1997b]. Higher levels of HCV RNA might be relatively underestimated by Amplicor Monitor assay compared to the TaqMan PCR assay. This underestimation might be caused by shortages of nucleotides and primers due to their exponential consumption during the later phase of PCR in the assay. Overall, the sensitivity was approximately equal in both assays.

When the sensitivities of these assays were compared with serotype 1 (Fig. 2B) or serotype 2 (Fig. 2C) separately, that of TaqMan PCR assay seemed superior to that of Amplicor Monitor assay with serotype 2. The different sensitivity in serotype 2 probably originates from mismatches between the PCR primers and the target HCV RNA in the Amplicor Monitor assay (two mismatches with serotype 2 sequence) [Hawkins et al., 1997]. A better assay for clinical studies might be developed by correcting the primer target mismatches in the Amplicor Monitor assay [Hawkins et al., 1997].

Unfortunately, only eight patients were treated with IFN (six sustained responders, two nonsustained responders). Although the six sustained responders tended to have lower levels of HCV RNA compared to the two nonresponders, the correlation of HCV RNA levels determined by TaqMan PCR assay and the outcome of IFN therapy could not be evaluated because the patients treated with IFN after the assay were too few. Further studies will be required to prove the usefulness of this method for prediction of the outcome of IFN therapy and for designing effective therapy schedules for individual patients.

ACKNOWLEDGMENTS

We thank Dr. Kouichi Tatsumi, Division of Biology and Oncology, National Institute of Radiological Sciences, for providing the opportunity to use PRISM 7700. We are grateful to Professor Masao Omata, Second Department of Medicine, Faculty of Medicine, Tokyo University, for helpful discussions and for encouraging us to perform this study.

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